

# CD28 Costimulation Is Required for In Vivo Induction of Peripheral Tolerance in CD8 T Cells

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## Abstract

Whereas ligation of CD28 is known to provide a critical costimulatory signal for activation of CD4 T cells, the requirement for CD28 as a costimulatory signal during activation of CD8 cells is less well defined. Even less is known about the involvement of CD28 signals during peripheral tolerance induction in CD8 T cells. In this study, comparison of T cell responses from CD28-deficient and CD28 wild-type H-Y-specific T cell receptor transgenic mice reveals that CD8 cells can proliferate, secrete cytokines, and generate cytotoxic T lymphocytes efficiently in the absence of CD28 costimulation *in vitro*. Surprisingly, using pregnancy as a model to study the H-Y-specific response of maternal T cells in the presence or absence of CD28 costimulation *in vivo*, it was found that peripheral tolerance does not occur in CD28KO pregnant mice in contrast to the partial clonal deletion and hyporesponsiveness of remaining T cells observed in CD28WT pregnant mice. These data demonstrate for the first time that CD28 is critical for tolerance induction of CD8 T cells, contrasting markedly with CD28 independence of *in vitro* activation, and suggest that the role of CD28/B7 interactions in peripheral tolerance of CD8 T cells may differ significantly from that of CD4 T cells.

Key words: pregnancy • clonal deletion • clonal anergy • B7 costimulatory molecules • cytotoxic T lymphocytes

## Introduction

The antigen specificity of T cells is determined by the TCR $\alpha\beta$  heterodimer. However, the ability of a T cell to be activated by TCR-mediated antigen recognition is regulated by additional signals such as those provided by cell surface costimulatory molecules. A critically important costimulatory pathway that functions in control of CD4 T cell activation is the highly studied CD28/CTLA-4/B7 pathway. T cells express cell surface CD28 that interacts with its ligands B7.1 or B7.2 expressed primarily on APCs such as macrophages, B cells, and dendritic cells (1). *In vitro*, TCR activation (signal one) in the absence of CD28 (signal two) has been shown to induce anergy, a state of unresponsiveness to subsequent encounters with antigen (2, 3), whereas TCR signaling in conjunction with costimulatory CD28 augments T cell activation by up-regulating mechanisms that enhance survival (bcl-2) and proliferation (IL-2 production; references 4–6). T cell responses are also modulated by a second B7 receptor, CTL antigen 4 (CTLA-4),\*

which delivers negative signals that down-regulate T cell activation (7, 8).

The role of the CD28/CTLA-4/B7 costimulatory pathway in CD4 T cell activation has also been analyzed in *in vivo* models. CD28KO mice are deficient in T cell-dependent antibody responses and in other responses dependent on CD4 Th activity (9). In contrast, CTLA-4 KO mice suffer from a lethal, massive lymphoproliferation of CD4 T cells driven by antigen, implicating CTLA-4 as critical for down-regulation of CD4 T cell activation (10, 11). A series of studies using adoptive transfer of OVA-specific class II-restricted TCR transgenic T cells has dissected the roles of CD28/CTLA-4/B7 pathways in immunity and tolerance *in vivo* (12–17). Inhibition of both CD28 and CTLA-4 signaling by administration of CTLA-4-Ig *in vivo* prevented priming of OVA-specific CD4 T cells. However, these T cells maintained their naive phenotype and were capable of subsequent response to antigen challenge, suggesting that TCR signaling alone could not drive T cells to become either activated or anergized (14). If only the B7/CTLA-4 pathway was inhibited, either with anti-CTLA-4 antibodies (14) or in CTLA-4-deficient mice (17), CD4 T cells were not only primed to antigen delivered in an immunogenic manner, but also to antigen presented in a tolero-

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\*Abbreviations used in this paper: CTLA, CTL antigen; TCM, tissue culture medium.

genic manner, compatible with a requirement for CTLA-4 in the induction of anergy in vivo. These data imply that signal 2 provided through CD28 drives full activation of CD4 T cells, whereas signaling through CTLA-4 results in a negative signal that can induce long-term antigen-specific tolerance.

Although the importance of CD28 signaling in CD4 T cell systems is well established both in vivo and in vitro, the role of CD28 in the activation of CD8 T cells is less clear. A number of in vitro systems have demonstrated CD28 independence of CD8 T cell activation (18–20) whereas others have shown that CD28 is required for optimal activation of CD8 cells (21–23). In vivo, it was found in one study that induction of LCMV-specific CTL activity was not detectably different in CD28-deficient and CD28 wild-type mice (9). Other groups have explored the role of CD28/B7 interactions in CD4-independent CD8 responses in vivo and have shown that while antigen-specific T cell expansion in CD28-deficient mice is indeed diminished, these cells are still capable of developing effector function and memory responses (24, 25).

The role of CD28 costimulation in induction of peripheral tolerance in CD8 T cells has not previously been assessed. To address this question, female CD28-deficient H-Y-specific TCR transgenic mice were compared with their CD28WT counterparts for the induction of tolerance to the H-Y antigen expressed by male fetuses during pregnancy. Surprisingly, while CD28 is dispensable for in vitro activation of CD28-deficient H-Y-specific T cells, it was found that CD28 is required for the induction of decreased antigen-specific proliferation and CTL generation as well as for clonal deletion of peripheral CD8 T cells.

## Materials and Methods

**Mice.** H-Y-specific TCR transgenic RAG-2 knockout (CD28WT H-Y) mice were generated as described previously (26) and maintained in our breeding facility at Bioqual Inc. (Rockville, MD). CD28WT H-Y mice were bred with mice deficient in CD28 (CD28KO; The Jackson Laboratory) to establish TCR transgenic RAG-2-deficient CD28-deficient (CD28KO H-Y) lines. H-Y mice were also bred to mice deficient for both B7-1 and B7-2 (B7DKO; gift of Arlene Sharpe, Harvard University, Cambridge, MA) to establish TCR transgenic RAG-2-deficient B7-1 and B7-2-deficient (B7DKO H-Y) lines. C57BL/6 mice were obtained from National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD).

Timed pregnant females were generated by mating females with syngeneic males for 18 h. The day the pair was separated was counted as day 0 (d0) of gestation. Pregnant females were killed at day 18 of gestation (d18), and spleen cell suspensions were prepared. Sex of the fetuses from each pregnancy was determined microscopically and the number of males/litter noted.

**Cell Culture.** Single cell suspensions were prepared and maintained in complete tissue culture medium (TCM) consisting of 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg streptomycin, 20 mM HEPES, 100 µM nonessential amino acids, 1 mM sodium pyruvate, and 25 µM 2-ME in RPMI 1640 (Bio-whittaker). Cell cultures were maintained at 37°C in 5% CO<sub>2</sub>.

**Cellular Proliferation Assay.** Spleen cells were washed extensively in 1× PBS to remove residual protein, then 10<sup>7</sup> spleen cells/ml were incubated in 1 µM CFSE (Molecular Probes) for 8 min at room temperature as described previously (27). The labeling reaction was quenched with an equal volume of FBS, the cells centrifuged, then washed three times in TCM. CFSE-labeled cells were diluted in TCM and were cultured at 4 × 10<sup>5</sup> in 96-well U-bottom plates for 72 h in the absence or presence of titrated H-Y peptide (KCSRNRQYL; reference 28). Alternatively, CFSE-labeled cells were cultured with 4 × 10<sup>5</sup> irradiated C57BL/6 male spleen cells (500R) as a source of H-Y-expressing APCs. Cultures were harvested at 72 h, stained with biotinylated anti-T3.70 (for the detection of the clonotypic H-Y-specific T cells [29]) for 30 min at 4°C, washed 3× in FACS<sup>®</sup> buffer (0.2% BSA, 0.01% sodium azide in HBSS without phenol red), then incubated with avidin-CyChrome for 10 min (BD Biosciences) followed by extensive washing in FACS<sup>®</sup> buffer. Immediately before analysis, cells were resuspended in Annexin-binding buffer (BD Biosciences) and labeled with Annexin-V-PE per manufacturer's instructions (BD Biosciences) with the exception that Annexin V was diluted 1:5 rather than used neat. Cells were analyzed on a FACScan<sup>™</sup> cytometer (BD Biosciences). 10,000 events in a live gate were acquired although all ungated events were saved for later analysis.

**Intracellular Cytokine Assay.** Spleen cells were labeled with CFSE and cultured with 500R-irradiated C57BL/6 male APCs, 1,000 nM H-Y peptide, or 1 nM H-Y peptide for 72 h. At 72 h, either 200 ng/ml PMA plus 750 ng/ml ionomycin or 1,000 nM peptide were added with GolgiStop (as per manufacturer's recommendation; BD Biosciences) to the cultures for an additional 5 h at 37°C in 5% CO<sub>2</sub>. Cultures were then harvested and labeled with T3.70-biotin followed by avidin-CyChrome as described above. Detection of intracellular cytokines was performed as described by manufacturer's instructions (BD Biosciences). Briefly, cells were fixed in Cytofix/Cytoperm solution for 20 min at room temperature, washed in PermWash buffer, then incubated with PE-labeled antibodies, anti-mouse IFN-γ, or the recommended isotype control for 30 min, followed by extensive washes in PermWash buffer before analysis on a FACScan<sup>™</sup>. 10,000 events gated on T3.70-positive cells were collected. The percentage of IFN-γ-expressing cells was calculated by subtracting the background staining observed with the isotype control from the percentage observed with the IFN-γ-specific antibody.

**Flow Cytometry-based CTL Assay.** Spleen cell suspensions were prepared and labeled with CFSE as described above. 4 × 10<sup>6</sup> CFSE-labeled spleen cells/ml were cultured for 72 h with H-Y peptide at 37°C in 5% CO<sub>2</sub>. At the end of cultures, cells were harvested and passed over Lymphocyte Separation Medium (ICN Biochemicals) to eliminate dead cells. Cells were then washed, resuspended in TCM, and counted. Flow cytometric analysis of this population revealed that >90% express the clonotypic TCR. These effector populations were then cultured with EL-4 targets in the absence or presence of 1 µM H-Y peptide for 4 h at various effector to target ratios. In some experiments, an irrelevant peptide (pigeon cytochrome C 88–104 (KAERADLIAY-LKQATAK; reference 30) was also used and showed no difference from EL-4 cells alone. At the end of 4 h, cultures were harvested, centrifuged, and resuspended in Annexin-binding buffer (BD Biosciences). Annexin-V-PE and propidium iodide were added per manufacturer's instructions (BD Biosciences). Analysis of target cell death was performed by gating on EL-4 cells (FSC<sup>hi</sup>CFSE<sup>neg</sup>) and assessing expression of Annexin V. Specific death was calculated by subtracting the percentage of Annexin

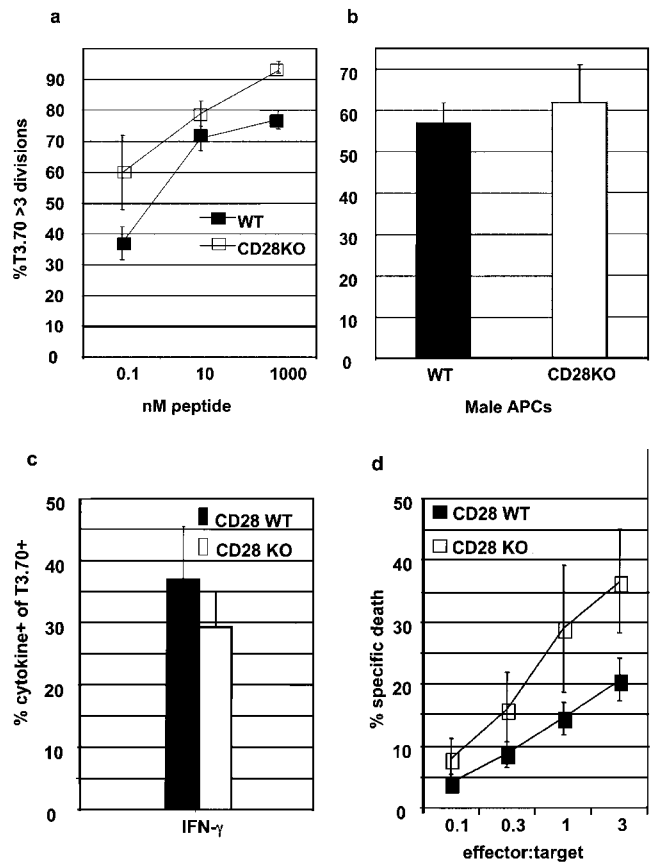
V-positive targets in culture with effectors but no peptide, from the percentage of Annexin V targets in cultures in which H-Y peptide was present.

## Results

*CD28 Is Not Required for Proliferation, IFN- $\gamma$  Production, or CTL Induction by H-Y-specific T Cells from TCR Transgenic Mice.* To assess the requirement for CD28 in class I-restricted T cell responses to antigen, we evaluated in vitro the responsiveness of TCR transgenic H-Y-specific T cells that either do or do not express CD28 (CD28WT H-Y or CD28KO H-Y). In all cases the TCR transgenic mice used in experiments are RAG-2 KO. Responsiveness was assessed by three criteria: proliferative responses, cytokine production, and CTL activity. Spleen cells from CD28WT or CD28KO H-Y females were labeled with CFSE and the proliferative responses to peptide or male APC stimulation evaluated by CFSE dye dilution. Fig. 1 illustrates proliferative responses of clonotype T3.70<sup>+</sup> T cells from H-Y CD28WT and CD28KO females in response to titrated doses of peptide. T cells from KO mice responded to both high (1,000 nM) and low dose (0.1 nM) peptide as well as T cells from WT mice. There was no proliferation to a nonspecific peptide (pCytC 88–104, unpublished data) confirming specificity of the proliferative response. Cultures from CD28KO H-Y mice demonstrated expansion of clonotypic T cells that was comparable to that from CD28WT cultures at the end of 72 h, indicating that CD28KO cells both proliferate and survive in response to antigen (unpublished data). Equivalent responses by CD28KO and CD28WT T cells were also observed when T cells were stimulated with male APCs, representing encounter with “physiological” levels of the H-Y peptide. (Fig. 1 b).

To further evaluate a role for CD28 costimulation in the generation of effector function in vitro, cytokine production and CTL activity were evaluated. The IFN- $\gamma$  response of CD28KO H-Y T cells was comparable to that of CD28WT H-Y T cells when activated with 1000nM peptide (Fig. 1 c) or with 1 nM peptide (see Fig. 3 c). H-Y-specific T cells from CD28WT or CD28KO mice were also assayed for H-Y-specific cytotoxic activity after 72 h of in vitro stimulation with 1  $\mu$ M H-Y peptide and revealed undiminished CTL generation in the absence of CD28 (Fig. 1 d). Thus, CD28-deficient T cells can proliferate efficiently in vitro to even low doses of antigen, and are not inhibited in their ability to generate CTL and IFN- $\gamma$  responses when compared with CD28-expressing T cells.

*In Vivo Encounter of Antigen during Pregnancy Results in a Partial Clonal Deletion of CD28WT but Not CD28KO H-Y-specific T Cells.* To determine the role that CD28 may play in an in vivo response to physiological “self” antigen, we next compared the ability of pregnancy to induce clonal deletion and altered T cell responsiveness in CD28WT and CD28KO mice. We have previously described that the number of H-Y-specific T cells in the spleen and lymph nodes of female TCR transgenic mice decreased in an anti-



**Figure 1.** CD28 is not required for H-Y-specific T cell proliferation, IFN- $\gamma$ , or CTL function.  $4 \times 10^5$  CFSE-labeled CD28WT or CD28KO H-Y spleen cells were cultured with (a) various concentrations of H-Y peptide or (b)  $4 \times 10^5$  C57BL/6 male spleen cells for 72 h, harvested, and analyzed for dye dilution after gating on T3.70<sup>+</sup> (anti-H-Y specific) Annexin V<sup>-</sup> cells. Results represent pooled data from multiple experiments (CD28WT,  $n = 28$ ; CD28KO,  $n = 10$ ). Cultures without antigen generally had <5% of cells with >3 divisions. (c) Absence of CD28 does not alter IFN- $\gamma$  production.  $4 \times 10^5$  spleen cells from CD28WT or CD28KO H-Y mice were cultured in the presence of 1,000 nM peptide for 72 h, then harvested and cultured for an additional 6 h in the presence of GolgiStop, 200 ng/ml PMA, and 750 ng/ml ionomycin. Similar results were obtained stimulating with peptide instead of PMA and ionomycin. Clonotypic T cells were analyzed for intracellular expression of IFN- $\gamma$ . Results represent pooled data from multiple experiments. (CD28WT,  $n = 8$ ; CD28KO,  $n = 8$ ). (d) Absence of CD28 does not inhibit the generation of CTL function in vitro.  $4 \times 10^6$  CFSE-labeled spleen cells from CD28WT or CD28KO H-Y mice were cultured with 1  $\mu$ M peptide for 72 h, harvested, and cultured with EL-4 cells in the absence or presence of peptide for 4 h, and EL-4 cells then analyzed for expression of Annexin V as an indicator of death. % specific death = % Annexin V-positive targets in the presence of peptide minus % Annexin V-positive targets without peptide. Results represent pooled data from multiple experiments. (CD28WT,  $n = 9$ ; CD28KO,  $n = 6$ ).

gen-specific manner during pregnancy (26). In the present study, when compared with nonpregnant controls, the number of clonotypic T cells decreased by  $\sim 50\%$  ( $4.2$  vs.  $2.0 \times 10^6$ ,  $P \leq 0.05$ ) in the spleens of CD28WT females at day 18 of pregnancy (d18) when male fetuses were present, but not when litters consisted only of female fetuses ( $5.3 \times 10^6$ ; Fig. 2 a). Recovery of non-T cells from d18 pregnant females was not significantly different from that observed

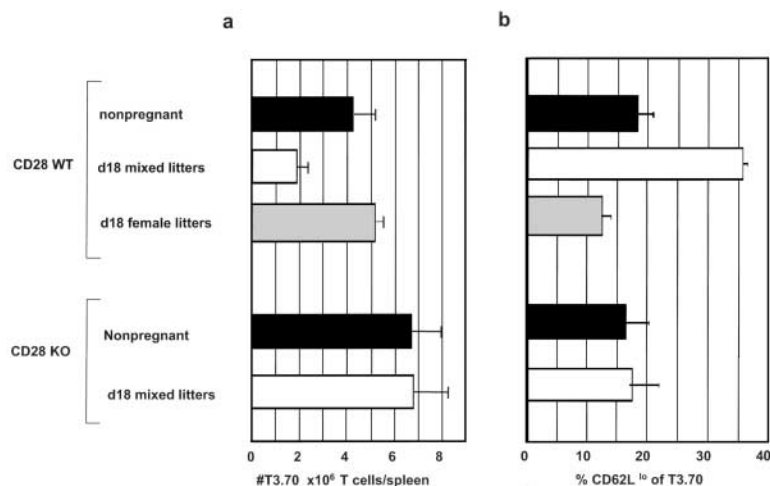
from nonpregnant females ( $19.1$  vs.  $17.4 \times 10^6$ ) or pregnant females carrying litters consisting only of females ( $20.2 \times 10^6$ ). In contrast, there was no significant decrease in clonotype-positive T cells in the spleens of pregnant CD28KO mice ( $6.8$  vs.  $6.7 \times 10^6$ ; Fig. 2 a), indicating that clonal elimination is CD28-dependent. Splenic size was comparable between nonpregnants from the WT and KO strain ( $23.3$  vs.  $27.2 \times 10^6$ ).

To further characterize the phenotype of H-Y-specific T cells from pregnant CD28WT and CD28KO H-Y mice, we analyzed levels of CD62L, an indicator of previous antigen encounter. Fig. 2 b reveals that the clonotypic T cells remaining in the d18 pregnant CD28WT females exhibit a significant increase in the percentage of T cells that are CD62L<sup>lo</sup> compared with T cells recovered from CD28WT females whose litters consisted only of females ( $35.9 \pm 0.8\%$  vs.  $12.4 \pm 1.6\%$ ,  $P \leq 0.01$ ) or nonpregnant females ( $18.3 \pm 2.8\%$ ; Fig. 2 b). In contrast, there was no increase in CD62L<sup>lo</sup> cells from pregnant CD28KO H-Y as compared with CD28KO H-Y nonpregnants (Fig. 2 b). There were no alterations in either TCR or CD8 levels in CD28KO T cells compared with CD28WT cells from either nonpregnant or pregnant mice (unpublished data).

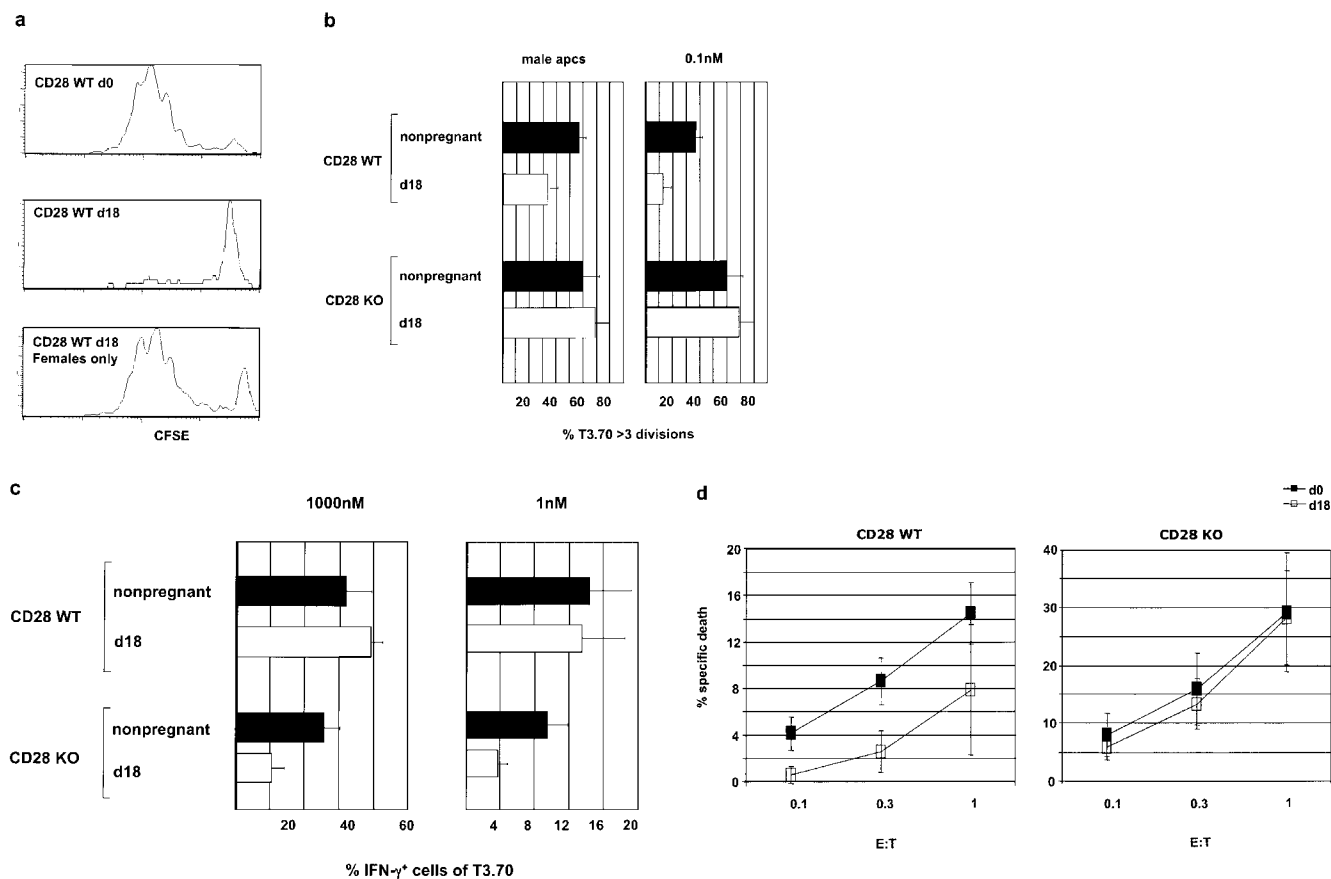
*In Vivo Encounter of Antigen during Pregnancy Results in Hyporesponsiveness in Remaining H-Y-specific T Cells in CD28WT but Not in CD28KO Mice.* We next addressed the ability of pregnancy and H-Y exposure to induce antigen-specific unresponsiveness in CD28KO mice. CFSE-labeled spleen cells from CD28WT or CD28KO H-Y mice were stimulated *in vitro* with peptide and assessed for proliferation of clonotypic Annexin V-negative cells. There was a significant decrease in the ability of the remaining T cells from d18 pregnant CD28WT H-Y females to proliferate in response to H-Y peptide when compared with nonpregnant controls or females pregnant with litters of only female fetuses (Fig. 3 a). When stimulated with  $0.1$  nM peptide, only  $13 \pm 5.3\%$  of T3.70 cells from CD28WT H-Y d18 pregnant females underwent 3 or more divisions, whereas  $37 \pm 5.4\%$  and  $63 \pm 14\%$  of T3.70 cells divided three or more times from CD28WT H-Y nonpregnant fe-

males or CD28WT H-Y pregnancies with all-female litters, respectively (Fig. 3, a and b;  $P < 0.05$ ). In contrast, clonotypic cells from CD28KO H-Y pregnant mice proliferated comparably to T cells from CD28KO H-Y nonpregnants ( $60 \pm 12\%$  vs.  $69 \pm 12\%$ ; Fig. 3 b). Similar results were obtained with male APCs as a source of antigen (Fig. 3 b). Cells from CD28WT H-Y d18 pregnant mice could be driven to divide to a degree similar to nonpregnant controls when challenged with a potent TCR stimulus such as high dose ( $1,000$  nM) peptide or anti-TCR antibodies (unpublished data, and reference 26). These results suggest that when class I restricted, H-Y-specific T cells encounter fetal H-Y antigen *in vivo*, both clonal deletion and the induction of hyporesponsiveness contribute to peripheral tolerance, and that both mechanisms are CD28 dependent.

To further address the phenotype and functionality of T cells from d18 pregnant CD28KO and CD28WT mice, we assessed two parameters of functional activation: cytokine production and CTL activity. Splenic T cells from nonpregnant or pregnant CD28WT H-Y and CD28KO H-Y females were compared for the ability to produce cytokine in response to low dose ( $1$  nM) or high dose ( $1,000$  nM) peptide. There was no decrease in IFN- $\gamma$ -producing clonotypic T cells from CD28WT H-Y pregnant mice compared with nonpregnants in response to either dose of antigen, in contrast to the diminished proliferative and CTL responses observed in these T cells (Fig. 3, b and d). Unexpectedly, however, there was a  $60\%$  decrease in IFN- $\gamma$  producing clonotypic T cells from d18 CD28KO H-Y pregnant mice compared with T cells from nonpregnant CD28KO H-Y females ( $3.8 \pm 1.1\%$  vs.  $9.6 \pm 2.3\%$ ,  $P < 0.05$ ) when stimulated with  $0.1$  nM peptide. Decreased responsiveness was not overcome using a high dose of antigen,  $1,000$  nM ( $12.1 \pm 4.2\%$  vs.  $29.5 \pm 5.5\%$ ,  $P < 0.02$ ). This altered IFN- $\gamma$  response indicates that encounter of the CD28KO T cells with H-Y antigen during pregnancy has occurred and that these T cells are not ignorant of the presence of H-Y. The absence of altered IFN- $\gamma$  production in cells from CD28WT pregnant females indicates that this is not a non-specific effect of pregnancy.



**Figure 2.** CD28 is required for induction of clonal deletion. (a) Decreased recovery of H-Y-specific T cells from pregnant females (WT nonpregnant,  $n = 28$ ; WT d18,  $n = 6$ ; WT d18 w/ females,  $n = 3$ ; KO nonpregnant,  $n = 16$ ; KO d18,  $n = 12$ ) and (b) increase in memory phenotype in remaining T cells (WT nonpregnant,  $n = 17$ ; WT d18,  $n = 6$ ; WT d18 females only,  $n = 3$ ; KO nonpregnant,  $n = 11$ ; KO d18,  $n = 11$ ). Spleen cells from WT or KO nonpregnant, or d18 pregnant with mixed litters or litters with only females were analyzed for expression of T3.70 (H-Y-specific TCR) and CD62L by flow cytometry. Results represent pooled data from multiple experiments.



**Figure 3.** (a) H-Y-specific T cells recovered from CD28WT d18 pregnant mice exhibit decreased proliferation to H-Y peptide.  $4 \times 10^5$  CFSE-labeled spleen cells from either nonpregnant or d18 pregnant H-Y CD28WT mice were cultured with 0.1 nM peptide for 72 h. T cells from pregnancies with mixed litters were compared with pregnancies with only females in the litter. (b) H-Y-specific T cells recovered from CD28KO pregnancies do not exhibit decreased proliferation to H-Y peptide or male APCs.  $4 \times 10^5$  CFSE-labeled spleen cells from either nonpregnant or d18 pregnant H-Y CD28WT or KO mice were cultured with 0.1 nM peptide for 72 h. Proliferation was assessed by dye dilution after gating on T3.70<sup>+</sup> Annexin V<sup>-</sup> cells (nonpregnant,  $n = 28$ ; d18,  $n = 6$ ; d18 females only,  $n = 3$ ; CD28KO nonpregnant,  $n = 16$ ; CD28WT d18,  $n = 12$ ). (c) Fewer H-Y-specific T cells recovered from CD28KO pregnancies secrete IFN- $\gamma$ .  $4 \times 10^5$  spleen cells from CD28WT or CD28KO H-Y mice were cultured in the presence of 1,000 nM or 1 nM peptide for 72 h, then harvested and cultured for an additional 6 h in the presence of GolgiStop, 200 ng/ml PMA, and 750 ng/ml ionomycin. Clonotypic T cells were analyzed for intracellular expression of IFN- $\gamma$ . Similar results were obtained in experiments restimulating with peptide instead of PMA and ionomycin. (d) H-Y-specific T cells from CD28KO d18 pregnant mice do not exhibit decreased CTL activity.  $4 \times 10^6$  CFSE-labeled spleen cells from CD28WT or CD28KO H-Y mice were cultured with 1  $\mu$ M peptide for 72 h, harvested, and cultured with EL-4 cells in the absence or presence of peptide for 4 h. EL-4 target cells were then analyzed for expression of Annexin V by flow cytometry. Results represent pooled data from multiple experiments (CD28WT nonpregnant,  $n = 9$ ; CD28WT d18,  $n = 3$ ; CD28KO nonpregnant,  $n = 6$ ; CD28KO d18,  $n = 4$ ).

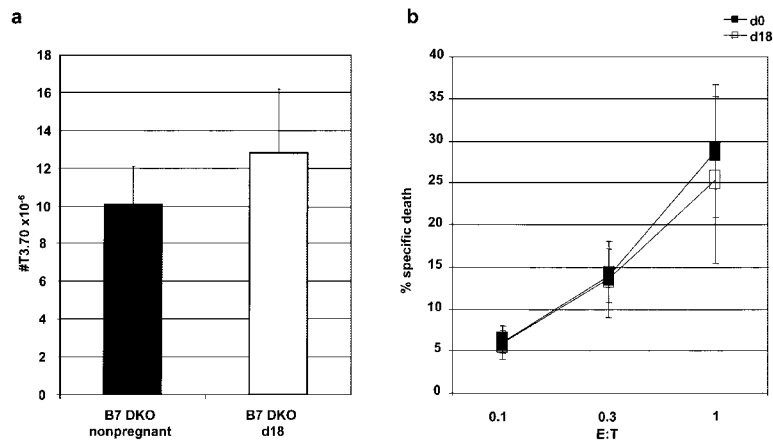
H-Y-specific CTL responses were also compared in spleen cells from pregnant or nonpregnant CD28WT and CD28KO H-Y mice. There was approximately a 50% decrease in CTL activity observed in T cells from pregnant CD28WT females compared with that observed from CD28WT nonpregnant females (Fig. 3 c). However, there was no alteration in the cytotoxic activity from cells recovered from pregnant CD28KO females compared with those recovered from CD28KO nonpregnant females. Collectively these data suggest that in the absence of CD28, pregnancy fails to induce either clonal deletion or hyporesponsiveness of H-Y specific CD8 T cells.

To determine whether the defect in CD8 tolerance observed in CD28-deficient mice reflects a requirement for CD28/B7 interactions, we generated H-Y mice that were deficient in both B7.1 and B7.2 (B7DKO). B7DKO H-Y

pregnant females did not undergo deletion of clonotypic T cells when compared with recovery from B7DKO H-Y nonpregnants (Fig. 4 a), paralleling the absence of deletion observed in T cells from the CD28KO H-Y pregnant mice. Furthermore, there was no alteration in CTL activity (Fig. 4 b) or proliferative responsiveness (unpublished data) of cells recovered from B7DKO pregnant mice. Thus, both deletion of clonotypic T cells and the induction of diminished responsiveness in surviving cells are dependent upon B7 as well as CD28, consistent with a requirement for CD28/B7 interaction in these pathways of peripheral tolerance.

## Discussion

This study has explored for the first time the role of CD28 in the induction of peripheral tolerance in CD8 T



**Figure 4.** B7 is required for induction of peripheral tolerance. (a) T cells from B7DKO H-Y pregnant females were compared with B7DKO H-Y nonpregnant females for T3.70 expression. (b) H-Y-specific T cells from B7DKO d18 pregnant mice do not exhibit decreased CTL activity.  $4 \times 10^6$  CFSE-labeled spleen cells from B7DKO H-Y mice were cultured with  $1 \mu\text{M}$  peptide for 72 h, harvested, and cultured with EL-4 cells in the absence or presence of peptide for 4 h. EL-4 target cells were then analyzed for expression of Annexin V by flow cytometry. Results represent pooled data from multiple experiments. (B7DKO nonpregnant,  $n = 3$ ; B7DKO d18,  $n = 3$ ).

cells in vivo. Analyzing proliferation, cytokine secretion, and CTL generation of MHC class I-restricted T cells from CD28WT and CD28KO H-Y RAG-2 KO mice, we have found that CD28 costimulation is dispensable for in vitro activation. Activation of CD28KO T cells was not diminished even when using very low concentrations of peptide antigen, or male APCs that express a low but physiological concentration of antigen. It was therefore surprising that we found CD28 is required in vivo for both the induction of clonal deletion and for antigen-specific hyporesponsiveness of CD8 T cells. These data demonstrate a previously unanticipated role for CD28 in tolerance induction of CD8 cells.

We have previously used pregnancy as a model to study peripheral tolerance in the maternal immune system to the fetal antigen, H-Y. Tolerance to H-Y expressed by male fetuses appears to be mediated by a combination of decreased survival of H-Y-specific T cells in vivo and decreased responsiveness of the remaining T cells as determined by in vitro challenge with antigen (26). That this is not a nonspecific effect of pregnancy itself but rather antigen-mediated is confirmed by the lack of deletion or hyporesponsiveness observed in pregnant females with litters consisting of only female pups. The reduction in clonotypic T cells could not be accounted for either by TCR down-regulation, or by migration to the sites of H-Y antigen expression, either the placenta itself or the draining lymph nodes of the placenta (our unpublished data). Therefore, it is likely that the decreased numbers of H-Y reactive T cells are due to cell death resulting from antigen encounter whether occurring in lymphoid tissues, or in nonlymphoid tissues after emigration.

The dependence on CD28 costimulation for induction of clonal deletion is unexpected. At least for CD4 T cells, it has been proposed that CD28 costimulation is required for clonal deletion of T cells to self-antigen because activation is required for activation-induced cell death to occur (31). The absence of clonal deletion in CD28KO mice would therefore be consistent with this model. However, it is important to consider that while CD4 T cells have a requisite dependence on CD28 for activation, it appears that the

H-Y-specific CD8 T cells studied here do not. Therefore, it is in this respect surprising that clonal deletion of these CD8 cells is CD28 dependent while activation is not. These results suggest a novel role for CD28 in determining the fate of CD8 T cells.

H-Y-specific T cells that escape deletion in pregnant CD28WT females exhibit a form of anergy defined by decreased proliferative responses that are not reversed by IL-2 (26). The remaining CD28WT T cells in pregnant mice exhibit decreased levels of CD62L, confirmation that these cells have encountered antigen, and also have decreased CTL activity in response to H-Y antigen. In marked contrast, H-Y-specific T cells from pregnant CD28KO mice did not exhibit either decreased proliferation or decreased CTL activity, nor did they down-regulate CD62L raising the possibility that these T cells have not been activated by antigen. However, T cells from the d18 CD28KO pregnant mice had significantly lower levels of IFN- $\gamma$ -producing cells compared with T cells from the CD28KO nonpregnant females, indicating that these cells are not “antigen ignorant” but have seen antigen in vivo and responded, albeit in an altered manner. The decrease in INF- $\gamma$ -producing cells is a not a nonspecific effect of pregnancy since T cells from d18 CD28WT pregnant mice do not exhibit decreased IFN- $\gamma$ -producing cells.

The phenomenon of “split tolerance” observed here in CD8 cells from pregnant CD28 WT mice, in which unresponsiveness was induced in some but not other parameters of T cell responses, has previously been reported in other experimental systems. Diminished proliferative response, but intact cytokine and/or CTL function, has been observed in vitro (32) as well as in in vivo systems of peripheral tolerance (33, 34). However, the means by which this type of split tolerance occurs is unclear. Interestingly, the inverse functional pattern is observed in T cells from CD28KO H-Y pregnant mice, which have normal CTL activity and proliferation yet decreased numbers of IFN- $\gamma$ -producing cells. Future studies will determine whether there are differences in TCR signaling in T cells from CD28WT and CD28KO H-Y pregnant mice that may account for functional differences in these populations, as has previously

been suggested in circumstances of split tolerance (34). One question that arises is whether the “split tolerance” that we as well as others have observed is truly “tolerance” since there is not a total abrogation of responsiveness in these systems. In the experimental system characterized here, the hyporesponsiveness observed by the residual T cells appears to be sufficient to prevent an immune response and rejection of the fetus, and therefore could be argued to be true functional tolerance.

The role of costimulation in the induction of anergy has previously been addressed in studies of CD4 T cells, and it has been proposed that CTLA-4/B7 interactions may be particularly important in the induction of anergy (14, 17). It has also been reported that two kinds of anergy can be induced in vitro, one that is induced by TCR stimulation in the absence of CD28 and is reversible by IL-2 and the other that is induced by CTLA-4 signals and is not reversible by IL-2 (35). The antigen-specific hyporesponsiveness that we have observed in H-Y-specific transgenic CD8 T cells resembles the second of these anergic states, in that it is not reversible by IL-2 (26). However, in our model of peripheral tolerance, anergy induction does not occur in CD28KO mice, despite the fact that CTLA-4/B7 interactions remain intact in these animals. This finding supports the observation by Frauwirth et al. that demonstrated CTLA-4 is not required for induction of in vivo anergy using CD8 TCR transgenic mice (36), again suggesting different roles for the CD28/CTLA-4/B7 costimulatory pathway between CD4 and CD8 T cells. The importance of CD28/B7 interactions in tolerance induction is further confirmed by the lack of both deletion and anergy induced in the B7DKO H-Y pregnant.

In conclusion, our data demonstrate that CD8 cells can respond efficiently to even low concentrations of antigen in the absence of CD28 costimulation in vitro. However, peripheral tolerance in vivo does not occur in the absence of CD28. To our knowledge this is the first in vivo assessment of the role for CD28 in peripheral tolerance induction of CD8 cells and the first evidence that CD28 is critical for tolerance induction of CD8 cells. These findings suggest that the role of CD28/CTLA-4/B7 interactions in peripheral tolerance of CD8 T cells may differ significantly from that for CD4 T cells and demonstrate that CD28 is involved in both clonal deletion and induction of hyporesponsiveness by encounter with peripheral self-antigen.

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